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1. Bau et al, NAR 22:2811-2816 (1994)
2. Dean et al., NAR 14:2229-2240 (1986)
3. Malter et al, Science 246:664-666 (1989)
4. Shaw et al., Cell 46:659-667 (1986)
5. van Aarsen et al., Plant mol. biol. 28:513-524 (1995)
6. Wilson et al., Nature336:396-399 (1998).

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A Conserved AU Sequence from the 3′ Untranslat d Region of GM-CSF mRNA Mediates Selective mRNA Degradation

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Summary

The mRNAs of transiently expressed genes frequently contain an AU-rich sequence in the 3′ untranslated region. We introduced a 51 nucleotide AT sequence from a human lymphokine gene, GM-CSF, into the 3′ untranslated region of the rabbit β-globin gene. Our experiments demonstrate that this caused the otherwise stable β-globin mRNA to become highly unstable in vivo. The instability conferred by the AU sequence in the mRNA was partially alleviated by treatment of the cells with cycloheximide. We propose that the AU sequences are the recognition signal for an mRNA processing pathway which specifically degrades the mRNAs for certain lymphokines, cytokines, and proto-oncogenes.

Introduction

Much is now known about the DNA sequence elements involved in transcriptional modulation in higher eukaryotes (for a review see Serfling et al., 1985), but relatively little is understood about sequences that could be involved in the determination of mRNA stability. It is clear that, in a variety of cell types, many mRNAs are short lived while others have half-lives of many hours (Singer and Penman, 1973; Berger and Cooper, 1975). An example of a relatively stable species is β-globin mRNA, with a half-life of greater than 17 hr (Aviv et al., 1976; Lowenhaupt et al., 1978; Volloch and Housman, 1981). In contrast, the mRNAs for fibroblast interferon and *c-fos* have half-lives of 30 min or less (Raj and Pitha, 1981; Greenberg and Ziff, 1984).

Attention has recently been focused on the differences in mRNA stability between transcripts of oncogenes and their normal cellular homologues. For example, *myc* genes isolated from certain B lymphoid tumor cells have often lost the first exon as the result of a reciprocal translocation which juxtaposes the *myc* gene and immunoglobulin heavy chain locus (Battey et al., 1983). The truncated *myc* genes transcribe a more stable mRNA than the full-length *c-myc* gene (Piechaczyk et al., 1985; Rabbits et al., 1985). Interestingly, the viral oncogene *v-myc* also lacks the 5′ exon of *c-myc*. The viral *fos* gene, *v-fos*, is capable of transforming rat fibroblast cells in vitro (Miller et al., 1984) whereas the normal *c-fos* gene cannot. Studies using hybrid genes made between *v-fos* and *c-fos* indicate that the 3′ segment from the *c-fos* gene causes a 2- to 3-fold lower accumulation of the hybrid RNA (Miller et al., 1984). Furthermore, deletion of a 67 bp sequence from the *c-fos* 3′ untranslated region converts it into a transforming gene (Meijlink et al., 1985). While this effect was not studied at the RNA level, it is possible that the deletion causes the *c-fos* mRNA to be more stable and thereby increases its oncogenicity. Studies using *c-fos*/β-globin gene fusions (Treisman et al., 1985) have also implicated the 3′ untranslated sequences of the *c-fos* gene in mRNA instability.

We recently observed that an AT sequence within the 3′ untranslated region of the gene for the lymphokine granulocyte-monocyte colony stimulating factor (GM-CSF) is highly conserved through evolution. GM-CSF stimulates the growth, differentiation, and activation of hematopoietic cells of myeloid lineage, including granulocytes, monocytes, and eosinophils. It is also a growth factor for early multipotential cells and erythroid precursors. The GM-CSF protein is transiently secreted by T cells following lectin or antigen activation (Kelso and Metcalf, 1985). Comparison of the human and murine GM-CSF cDNA sequences indicated, surprisingly, that the most conserved region is a sequence composed exclusively of A and T nucleotides. It is located in the 3′ untranslated region and is 51 bp long in the human cDNA and 59 bp long in the mouse cDNA (Wong et al., 1985; Gough et al., 1984). These sequences are 93% conserved, while the coding regions are conserved to only 65%. We subsequently noted that similar long runs of A and T occur in the 3′ untranslated regions of numerous lymphokine, cytokine, and proto-oncogene mRNAs. The high degree of conservation implied functionality, and we therefore designed experiments to test whether the AT sequence has a definable role in gene expression. We synthesized the sequence from human GM-CSF and inserted it into the 3′ untranslated region of the rabbit β-globin gene. We show here that the AT sequence operates at the posttranscriptional level to specifically decrease the accumulation of globin transcripts. This effect is caused by marked reduction in mRNA stability. We propose that such AU sequence motifs in mRNAs are generally involved in the selective degradation of transiently expressed messengers.

Results

GM-CSF mRNA Is Unstable in T Lymphocytes

Expression of the GM-CSF activity by lectin-stimulated T lymphocytes is known to be transient (Kelso and Metcalf, 1985), but the stability of the GM-CSF mRNA has not been investigated. Therefore, we treated either lectin-stimulated or phorbol-diester-stimulated human T lymphoblastoid cells (from the cell line C10MJ-2; Ayra et al., 1984) with actinomycin D and examined the GM-CSF mRNA by Northern blot analysis at various times after treatment. As shown in Figure 1 (lanes 1–5), the level of GM-CSF mRNA in phytohemagglutinin (PHA) treated cells declined rapidly with a half-life of less than 30 min. In contrast, the relatively abundant GM-CSF mRNA in TPA (12-0-tetradecanoylphorbol-13-acetate) treated cells did not decay significantly over the two hour time course studied (Figure 1, lanes 6–10). We were interested to ob-

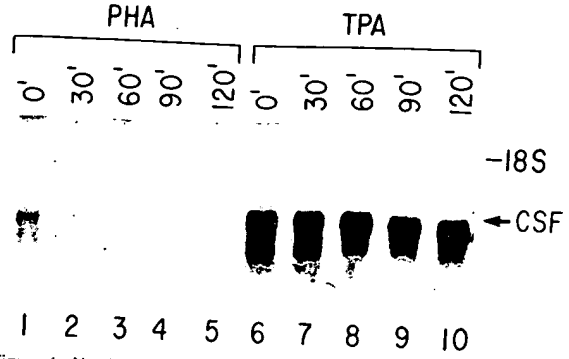


Figure 1. Northern Blot Analysis of GM-CSF mRNA Isolated from Actinomycin-D-Treated C10MJ-2 Cells

Human T lymphoblast cells (C10MJ-2) were induced for 2 hr with 0.3% phytohemagglutinin (PHA) (lanes 1–5) or 5 ng/ml TPA (lanes 6–10). Actinomycin D was then added to 5 μg/ml. Total cellular RNA was prepared at the indicated timepoints. Equal amounts of RNA (10 μg per lane) were run on a denaturing 1% agarose gel, transferred to nitrocellulose, and hybridized with a GM-CSF cDNA probe. 18S: 18S ribosomal RNA. CSF: GM-CSF mRNA.

serve that the GM-CSF mRNA produced by phorbol-diester-induced T cells is very stable. This could reflect the modulation by phorbol diesters of a specific mRNA degradation pathway.

The experiment shown in Figure 1 demonstrates that the GM-CSF mRNA transiently produced by lectin-treated T cells is unstable. We hypothesized that the AU-rich sequence in the 3′ untranslated region of the GM-CSF mRNA might play a role in determining its stability. This hypothesis was tested by characterizing the expression of a chimeric rabbit β-globin gene containing the putative destabilizing element from the human GM-CSF cDNA.

Insertion of an AU-Rich Sequence into the 3′ Untranslated Region of the Rabbit β-Globin mRNAs

The AT sequence of human GM-CSF was synthesized using complementary oligonucleotides (58-mers) with 5′ GATC overhanging ends, compatible with those generated by BglII cleavage. As a control, another sequence of the same length (but with 14 G's and C's interspersed among the sequence) was synthesized. In separate constructs, each synthetic duplex DNA was inserted at a BglII site in the 3′ untranslated region of the genomic rabbit β-globin gene (van Ooyen et al., 1979). Constructs having the correct orientation were identified using junction oligonucleotide probes and subsequently sequenced. The resulting β-globin constructs β-globin^{AT} and β-globin^{GC} are shown in Figure 2A. Construct β-globin^{AT} contains the AT sequence and β-globin^{GC} the control oligonucleotide with interspersed G and C nucleotides. The globin genes are included within a plasmid in which a polyoma virus enhancer element is located downstream of the gene to activate transcription from the β-globin promoter (deVilliers et al., 1982).

To test the effect of the AT sequence on gene expression, the two constructs, β-globin^{AT} and β-globin^{GC}, as well as the parental globin plasmid, were transfected into subconfluent NIH3T3 cells. Thirty hours later, cytoplasmic

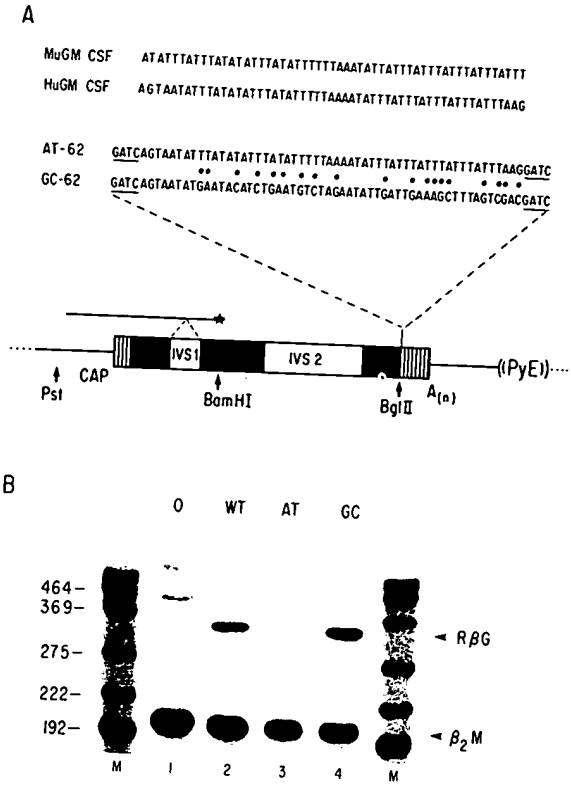


Figure 2. Transient Expression of β-Globin Genes Containing the AT Sequence from Human GM-CSF or a Control Sequence with G's and C's Interspersed

(A) The top two lines represent the conserved sequences occurring in the 3′ noncoding region of the mouse and human GM-CSF genes. AT-62, which contains the human GM-CSF sequence, was chemically synthesized as two complementary oligonucleotide 58-mers with 5′ cohesive ends (underlined). The double-stranded oligonucleotide GC-62 was synthesized as a control sequence. Nucleotide differences between AT-62 and GC-62 are indicated by dots. The synthetic sequences were inserted into the BglII site located in the 3′ noncoding region of the rabbit β-globin gene in the orientation as shown. The resulting constructs, pRβG^{AT} and pRβG^{GC}, contain the AT-62 or GC-62 sequence respectively. The diagram of the rabbit β-globin gene shows the exons (black), intervening sequences (IVS), and flanking sequences (striped). The DNA probe (453 nucleotides) used for S1 nuclease mapping was derived from a β-globin gene lacking IVS1 and is schematically depicted above the gene.

(B) S1 nuclease mapping of β-globin mRNA isolated from NIH3T3 cells 30 hr after transfection with the β-globin test DNAs. Equal amounts of total cytoplasmic RNA (50 μg) were analyzed. Mouse β_2 microglobulin mRNA levels were used as an internal standard. Lane 1: no DNA. Lane 2: wild-type β-globin DNA. Lane 3: pRβG^{AT}. Lane 4: pRβG^{GC}. M: marker of polyoma virus DNA digested with DdeI. The RβG and β_2M arrows indicate the fragments protected by correctly initiated and spliced globin mRNA (354 nucleotides) or β_2 microglobulin mRNA (205 nucleotides) respectively.

RNA was prepared and assayed by S1 nuclease protection analysis. Two DNA probes were used: a β-globin probe protecting correctly initiated and spliced β-globin mRNAs (see Figure 2A) and a mouse β_2 microglobulin probe (Parnes and Seidman, 1982). The latter was used as an internal standard to control the amount of RNA in each sample. As can be seen in Figure 2B, the level of accumulated β-globin^{AT} mRNA was drastically reduced compared with that of β-globin^{GC} and wild-type β-globin mRNAs (compare lane 3 with lanes 2 and 4). Densitomet-

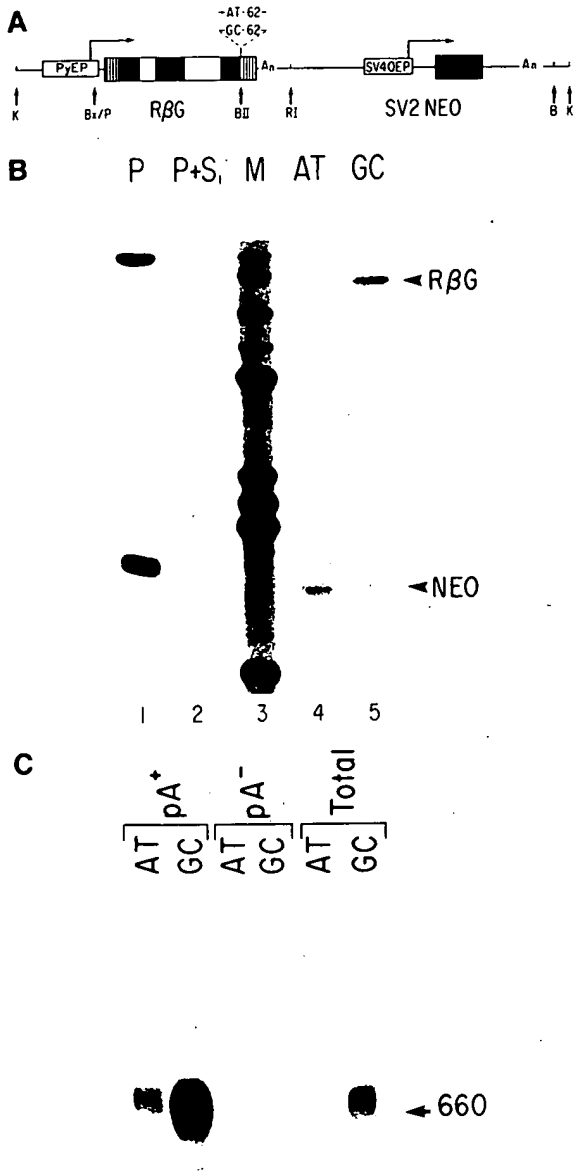


Figure 3. Expression of Modified β-Globin Genes in Stably Transformed Cell Populations

(A) Schematic representation of the DNA vectors, pNEORβG^{AT} and pNEORβG^{GC} used to establish NIH3T3 derived cell lines (TSWOP) with stably integrated copies of the rabbit β-globin^{AT} or rabbit β-globin^{GC} test gene (see Figure 2A) linked to SV2Neo DNA. Polyoma virus early promoter, PyEP, and SV40 early promoter, SV40EP, are shown with transcription start sites indicated by horizontal arrows. Protein coding sequences are shown as solid boxes. Restriction sites shown are to delineate subcomponents of the vector. K = KpnI, BX = BstXI, P = PvuII, BII = BglII, RI = EcoRI, B = BamHI.

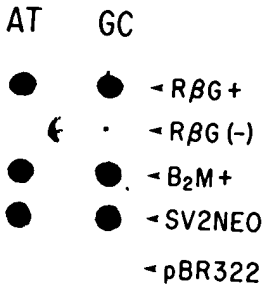
(B) S1 nuclease mapping of total cytoplasmic RNA isolated from TSWOP cell lines containing an integrated copy of pNEORβG^{AT} or pNEORβG^{GC} DNA. Lane 1: single-stranded DNA probes for rabbit β-globin mRNA (RβG; 453 nucleotides) or SV2neo mRNA (NEO, 82 nucleotides). Lanes 2, 4, and 5: S1-nuclease-resistant DNA probes after hybridization with 35 μg RNA from TSWOP cells (lane 2), 25 μg RNA from the pNEORβG^{AT}-containing cell line (lane 4), or 12.5 μg RNA from the pNEORβG^{GC}-containing cell line (lane 5). Lane 3: marker DNA fragments.

ric scanning indicated that the β-globin^{AT} mRNA level was less than 3% of the control. The accumulated levels of β-globin^{GC} and wild-type β-globin mRNA were similar. Additional transfection experiments were done in human HeLa, mouse 3T6, and L 929 cells. In all cases, the expression of the β-globin^{AT} construct resulted in 10- to 30-fold lower steady-state β-globin mRNA levels as compared with controls (data not shown).

The Decrease in Steady-State Levels of β-Globin^{AT} mRNA Is Not Due to Reduced Template Copy Number or a Lower Rate of Transcription

The observed decrease in steady-state β-globin^{AT} mRNA levels can possibly be explained by a lower gene copy number of β-globin^{AT} DNA than β-globin^{GC} DNA. Differences in transfection efficiencies could conceivably cause this. To control for this possibility, new vectors, pNEORβG^{AT} and pNEORβG^{GC}, were constructed. A schematic representation is shown in Figure 3A. In these constructs the β-globin promoter was replaced by the polyoma virus early promoter and a selectable marker gene transcription unit, SV2NEO (Southern and Berg, 1982), was linked downstream of the β-globin transcription unit. The linkage of SV2NEO not only provides a reference transcript to normalize copy number, it also allows the establishment of cell lines bearing integrated copies of the β-globin constructs. Accordingly, the two vectors bearing either β-globin^{AT} or β-globin^{GC} constructs were transfected as KpnI linear fragments into an NIH3T3 derived cell line and cells were selected for G418 resistance. Multiple resistant colonies were pooled to ensure a population of cells with a heterogeneous collection of integration sites. Subsequent analysis demonstrated that equivalent amounts of RNA from each cell population contained equivalent levels of SV2NEO transcripts; this indicates that the vector DNA copy number is approximately the same for both cell populations. An assay of mRNAs from the stably transformed cells which contains either pNEORβG^{AT} or pNEORβG^{GC} DNAs is shown in Figure 3B. Using a 2-fold greater amount of RNA from pNEORβG^{AT}-containing cells, barely detectable levels of β-globin^{AT}-specific transcripts were observed (Figure 3B, lane 4). Densitometer scans indicate that the level of β-globin^{AT} mRNA is approximately 3% that of β-globin^{GC} mRNA. This is in agreement with the findings of the transient transfection experiments. The accumulated mRNA levels in the 3T3 cell populations were also examined by Northern blot analysis. Figure 3C demonstrates that polyadenylated mRNAs of the correct size were present in both cell populations and that the level of β-globin^{AT} mRNA was drastically reduced, as expected from the S1 nuclease protection experiments. No significant level of nonpolyadenylated transcript could be detected in either instance (see Figure 3C).

(C) Northern blot analysis of β-globin mRNA produced by the pNEORβG^{AT} or pNEORβG^{GC} cell lines. Total cytoplasmic RNA was isolated, fractionated into poly(A)$^+$ and poly(A)$^-$ RNA. Poly(A)$^+$ (4 μg), poly(A)$^-$ (20 μg), or unfractionated RNA (20 μg) was electrophoresed through a denaturing agarose gel, transferred to nitrocellulose, and hybridized to a rabbit β-globin DNA probe.



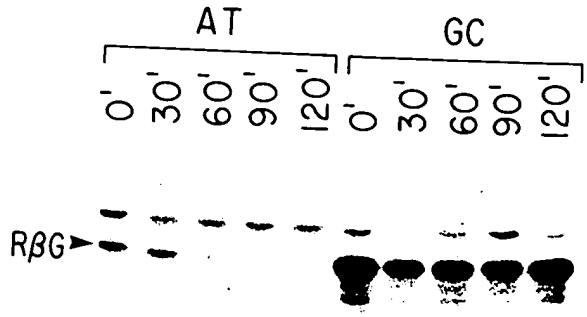


Figure 4. Analysis of β-Globin^{AT} and β-Globin^{GC} Transcription Rates Using a Nuclear Run-On Transcription Assay

RNAs were elongated in vitro in the presence of α-^{32}P-UTP, using nuclei isolated from pNEORβG^{AT} (AT) or pNEORβG^{GC} (GC) cell populations. The labeled RNAs were isolated and hybridized to the following DNAs immobilized on nitrocellulose filters. M13 single-stranded DNA (2.5 μg) containing an anti-sense strand insert of rabbit β-globin DNA (RβG^{+}), a sense strand (RβG^{-}) or anti-sense strand insert of mouse β_2 microglobulin DNA (β_2M^{-}), and double-stranded, denatured SV2NEO and pBR322 plasmid DNAs.

To determine if there are differences in the rates of transcription between the β-globin^{AT} and β-globin^{GC} transcription units, nuclear run-on experiments were performed. Transcription of the SV2NEO gene served as an internal control in these experiments as did that of β_2 microglobulin. The results, shown in Figure 4, indicate that the polymerase densities and presumably, therefore, the rates of transcription, are the same for both β-globin constructs. We conclude that the decreased steady-state level of β-globin^{AT} mRNA is most probably the result of a posttranscriptional event.

β-Globin^{AT} mRNA Decays Faster than β-Globin^{GC} mRNA

Having demonstrated that the presence of the AT sequence in the rabbit β-globin transcription unit does not decrease its rate of transcription, we proceeded to study its effect on mRNA stability. To measure the half-lives of β-globin^{AT} mRNA and β-globin^{GC} mRNA, the cell populations carrying integrated copies of the test vectors were treated with actinomycin D. Cytoplasmic mRNAs from the two cell populations were assayed at various times after treatment. The results of the S1 nuclease protection analysis, shown in Figure 5, indicate that β-globin^{AT} mRNA decays with a half-life of less than 30 min to a nearly undetectable level after 1 hr (lanes 1–5), whereas β-globin^{GC} mRNA remains stable for the 2 hr period (lanes 6–10). The SV2NEO reference transcript also remained stable during this period. Tritiated uridine pulse-chase experiments performed using stably transformed mouse L929 cells confirmed that β-globin^{AT} mRNA decayed at a greater rate (t½ less than or equal to 30 min) than the stable β-globin^{GC} control mRNA (data not shown). These results together suggest that the presence of the AU sequence in the β-globin mRNA specifically confers instability. The magnitude of this effect is sufficient to explain the reduction in accumulation levels of β-globin^{AT} mRNA.

Figure 5. Actinomycin D Treatment of Cells Reveals a Difference in Decay of β-Globin^{AT} and β-Globin^{GC} mRNAs

S1 nuclease analyses were performed on total cytoplasmic RNA samples (50 μg per lane) extracted from cell populations containing pNEORβG^{AT} (lanes 1–5) or pNEORβG^{GC} (lanes 6–10) DNA at indicated times (minutes) after treatment of the cells with 5 μg/ml actinomycin D. Faint bands at the top of the gel represent β-globin probe protected from S1 nuclease digestion by contaminating probe template. The arrows RβG and Neo indicate the S1-nuclease-resistant fragments generated by β-globin and SV2NEO transcripts respectively.

β-Globin^{AT} mRNA Is Stabilized in Cells Treated with the Translational Inhibitor Cycloheximide

Several studies on intrinsically unstable mRNAs have indicated that drugs which inhibit protein synthesis increase their stability (Raj and Pitha, 1981; Linial et al., 1985; Elder et al., 1984; Greenberg et al., 1986). This effect of protein synthesis inhibitors can occur at both the transcriptional and posttranscriptional levels.

We have tested the effect of cycloheximide, a compound that blocks peptide bond formation, on the cell populations containing either the pNEORβG^{AT} or pNEORβG^{GC} DNAs. As shown in Figure 6, the accumulation of β-globin^{AT} mRNA in the cytoplasm is greatly increased by cycloheximide treatment; β-globin^{AT} mRNA accumulated to levels approaching nearly 50% of β-globin^{GC} mRNA levels (compare lanes 1 and 2). There was a general increase in the level of all transcripts measured in cycloheximide-treated cells as compared with untreated cells. This likely reflects a transcriptional superinduction phenomenon which has been noted by others (Greenberg et al., 1986).

Figure 6. Effect of Cycloheximide Treatment on β-Globin^{AT} and β-Globin^{GC} mRNA Stability

Lanes 1 and 2: cell populations containing pNEORβG^{AT} (AT) or pNEORβG^{GC} (GC) DNA were treated for 3 hr with cycloheximide (10 μg/ml). Total cytoplasmic RNA was isolated and analyzed (50 μg per lane) by S1 nuclease mapping. Lane 3: size marker as described in Figure 2B.

Also, the appearance of aberrently initiated and spliced β-globin transcripts (deVilliers and Schaffner, 1981) was more pronounced in the presence of cycloheximide. However, in comparing the ratios of β-globin transcripts with SV2NEO transcripts, it is clear that the predominant effect of cycloheximide is on the stability of the β-globin^{AT} mRNA. When normalized to SV2NEO transcripts, the β-globin^{GC} mRNA increased about 2-fold, whereas β-globin^{AT} mRNA increased at least 25-fold.

Discussion

Our results demonstrate that the insertion of a 51 bp AT sequence, identical to that found in the human GM-CSF gene, into the 3′ untranslated region of the β-globin gene reduces cytoplasmic mRNA accumulation to approximately 3% of wild-type levels. The similar insertion of a control sequence, equal in length but containing G's and C's interspersed among the A's and T's, causes no detectable change in the cytoplasmic mRNA accumulation levels as compared with wild-type levels. This effect was observed in the two mouse (L929, NIH3T3) and one human (HeLa) cell lines tested. Analysis of the transcription rates for the β-globin^{AT} and β-globin^{GC} test constructs using a nuclear run-on assay indicated that both constructs were transcribed at equivalent rates. This finding suggested that the reduced accumulation levels of β-globin^{AT} mRNA might be due to a posttranscriptional event, and led us to study the stability of the two mRNAs. Analysis of the decay rate of β-globin^{AT} and β-globin^{GC} mRNAs, after treating cells with actinomycin D, revealed that a greater rate of decay occurred in the case of β-globin^{AT} mRNA. The half-life was estimated to be less than 30 min for β-globin^{AT} mRNA whereas the β-globin^{GC} mRNA showed no significant decay 2 hr after actinomycin D treatment. This difference is sufficient to explain the difference in cytoplasmic mRNA accumulation levels observed between the two constructs.

We have also measured the half-life of human GM-CSF mRNA under similar conditions in lectin-stimulated human T cells (using the C10MJ-2 line) and observed that it was less than 30 min. Thus, transfer of the AT sequence from the 3′ untranslated region of the GM-CSF gene to that of the rabbit β-globin gene renders the normally stable β-globin mRNA as unstable as the transiently expressed GM-CSF messenger.

AU sequences related to those in the GM-CSF 3′ untranslated region also occur in the 3′ untranslated region of other mRNAs. Figure 7 shows the AU-rich sequences

Hu GM-CSF	UAAUAUUUAUAUAUUUAUAUUUUUUAAAAAUAUUUAUUUAUUUAUUUAUUUAA
Mu GM-CSF	UUUAUUUAUAUAUUUAUAUUUUUUAAAAUAUUAUUUAUUUAUUUAUUUAUUU
Hu α IFN	AUUUGUUCUUUAUUUAUUUAUUUAUUUAUUUAUUUAUUUAA
Hu α IFN	UAUUUAUUUAUUUAA
Hu β IFN	UUUUGAAAUUUUUUAUUAAAUUAUGAGUUAUUUUUAUUUAUUUAAAUUUUAUUUUGGAAAA
Mu β IFN	UUUUUAUUAAUUUAUAAUUUAAAUUAUUUUCUACUUUUUAUUUAAACUUUUUAA
Hu γ IFN	UAUUUAUUAAUAUUUAACAUUAUUUAUAU
Hu IL 1	UUAUUUUUUAAUUAUUAUUUAUAUAUGUAUUUAUAAAAUAUAUUUAAAGAUAAUUAUAAUAU
Ra IL 1	AUUAUUUAUAUGUAUGUAUUUAUAAAUGUAUUUAAGAUAAUAAUUAUUAUUAUAUUUUAUA
Hu IL 2	UAUUUAUUUAAAUAUUUAAAUUUUUAUAUUUAUU
Mu IL 2	UAUUUAUUUAAAUAUUUAACUUUAAUUUAUUUUU
Mu IL 3	UAUUUAUUUAUGUAUUUAUGUAUUUAUUUAUUUAUU
Hu TNF	AUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUUA
Mu TNF	UUAUUAUUUAUUAUUUAUUUAUUAUUUAUUUAUUU
Hu LYM	AAAAAAAAUUAAAUUAUUUAUUUAU
Hu G-CSF	UAUUUAUCUCUAUUUAAUAUUUAUGUCUAUUUAA
Hu IgG1 IF	AAUUUUUAAUGGUUUUAUUUUUAAUAUUUAUAUAUUUAUAAUU
Hu cFOS	GUUUUUAAUUUAUUUAUUAAGAUGGAUUCUCAGAUAUUUAUAUUUUUAUUUUAUUUUUUU
Hu cSIS	UUUCCUUUUAUUUUUUAAAUGUAAAAUUUAUUUAUAUUUCGUAUUUAAA
Hu cMYC	UAAUUUUUUUUUAUUUAAGUACAUUUUGCUUUUUAAAGUUGAUUUUUUUCUAUUGUUUUUA
Hu cMYB	AUUUUUUAAAAAAAAAUAAAAUGAUUUAUUUGUAUUUUA

Figure 7. AU-Rich Sequences Found in the 3′ Untranslated Region of Several Lymphokine and Proto-Oncogene mRNAs

Abbreviations: Hu = human; Mu = murine; Ra = rabbit; GM-CSF = granulocyte-monocyte colony stimulating factor; αIFN = leukocyte interferon; β-IFN = fibroblast interferon; γIFN = lymphoblast interferon; IL1 = interleukin 1; IL2 = interleukin 2; IL3 = interleukin 3; TNF = tumor necrosis factor; Lym = lymphotoxin; IgG IF = IgG1 induction factor; G-CSF = granulocyte colony stimulating factor; cFOS = *fos* proto-oncogene; cSIS = *sis* proto-oncogene; c-MYC = *myc* proto-oncogene; c-MYB = *myb* proto-oncogene. The underlined/overlined sequence (AUUUA) is the largest motif common to all mRNAs shown. References: HuGM-CSF (Wong et al., 1985); MuGM-CSF (Gough et al., 1984); Mu α-Ifn (Shaw et al., 1983); Hu α-Ifn (Goeddel et al., 1981); Hu β-Ifn (Ohno et al., 1981); Mu β-Ifn (Higashi et al., 1983); Hu γ-Ifm (Gray et al., 1982); Hu IL-1, RaIL-2 (Furutani et al., 1985); Hu IL-2 (Kashima et al., 1985); MuIL-2 (Fuse et al., 1984); MuIL3 (Miyatake et al., 1985); Hu TNF, Hu LYM (Nedwin et al., 1985); MuTNF (Fransen et al., 1985); Hu G-CSF (Nagata et al., 1986); Hu IgGIF (Noma et al., 1986); Hu *c-fos* (van Straaten et al., 1983); Hu *c-sis* (Ratner et al., 1985); Hu *c-myc* (Battey et al., 1983); Hu *c-myb* (Gonda et al., 1985).

present in the mRNAs of several other lymphokines, cytokines, and proto-oncogenes. Most of these mRNAs are transiently xpressed. The conservation of an eight nucleotide AT-rich motif has also been noted for genes specifying inflammatory mediators (Caput et al., 1986), which includes many of the genes shown in Figure 7. Such long AT sequences have not been identified in the coding or noncoding regions of genes known to express stable transcripts. Of the 21 sequences shown, four are proto-oncogenes which are reported to be regulated by post-transcriptional mechanisms. For example, *c-myb* mRNA stability is increased following serum stimulation of chicken embryo fibroblasts. The variations in *c-myb* mRNA levels during cellular activation and proliferation seem to be predominantly the result of posttranscriptional control (Thompson et al., 1986). In the case of *c-fos*, the deletion of the AT-rich sequence confers transforming activity to the *c-fos* proto-oncogene when assayed in cultured rat fibroblast cells (Meijlink et al., 1985). To our knowledge, no other attempt has been made to delete the AT-rich sequences from any of the other genes shown.

The control sequence present in β-globin^{GC} mRNA changes 18 of the 51 nucleotides in the AU sequence present in β-globin^{AT} mRNA. Fourteen of those changes introduced G or C nucleotides into the sequence (Figure 1A). The control sequence does not contain the motif, AUUU, which occurs eight times in the AU sequence. It is not clear from experiments done to date precisely what minimal sequence is required to generate the destabilizing effect. Analysis of the sequences in Figure 7 shows that the largest sequence motif common to all of these genes is AUUUA. However, the most striking feature shared by all of these sequences is the occurrence of a single adenosine nucleotide followed by a polyuridine tract of three or more U's. The potential secondary structure generated by the AU sequence is unknown; there are no obvious inverted repeats in this sequence, but the potential base pairing of the poly(A) tail with the short poly(U) tracts cannot be ruled out with present data. Furthermore, although there could be a positional requirement of the AU sequences relative to the coding body or poly(A) tail, analysis of the sequences found in the 21 mRNAs shown in Figure 7 would predict a nonstringent positional requirement within the 3′ untranslated region. This remains to be demonstrated experimentally.

There are several possible mechanisms that could explain the effect of the AU sequence. For example, the sequence might alter the transport efficiency of nascent mRNA out of the nucleus to the cytoplasm. However, no difference between cytoplasmic RNA and total RNA has been observed to substantiate such a possibility. It is also conceivable that the AT sequences could function somewhat similarly to a TATA box to promote transcription of the opposite DNA strand, resulting in anti-sense RNA. No such transcripts wer observed in the nuclear run-on experiments, nor would the generation of such anti-sense transcripts be compatible with the results seen upon treatment of cells with cycloheximide. The AU sequences might interact with small nuclear RNPs (snRNPs), which have been implicated in other RNA processing steps such as splicing, 3′ terminus generation, and polyadenylation (reviewed by Birnstiel et al., 1985). The AU sequences bear some resemblance to sequences found downstream of many polyadenylation sites and could conceivably be causing premature termination or aberrant 3′ terminus generation. No evidence for either truncated or poly(A)$^-$ globin mRNA was observed by Northern blot analysis (see Figure 3C).

Our results are most consistent with the notion that the AU sequence causes the rapid loss of mature mRNAs in the cytoplasm. The sequence might direct the mRNA to a cytoplasmic compartment where rapid degradation occurs. Alternatively, it could be that the sequence is recognized by an RNA-binding protein involved in the specific degradation of transiently expressed mRNAs. Such a protein could be a processing endonuclease. Studies on isolated polysomes have indicated that the 3′ untranslated regions of mRNAs are particularly accessible to nucleases (Bergmann and Brawerman, 1980). In collaboration with Ian Kerr and colleagues (ICRF, London), we investigated the possible involvement of the 2–5 oligo adenylate dependent ribonuclease (Brown et al., 1976; Wreschner et al., 1981; also termed RNAase L, Lengyel, 1982) in the degradation of β-globin^{AT} mRNA, but were unable to find evidence for the involvement of this particular enzyme.

The finding that cycloheximide treatment can stabilize the β-globin^{AT} mRNA, enabling cytoplasmic accumulation to over 50% of control levels, suggests two possible mechanisms. Either a labile protein is involved in the degradation process and it decays during the treatment period, or the degradation of the mRNA requires its ongoing translation on polysomes. A rapid effect of the protein synthesis inhibitor emetine to increase *c-myc* accumulation, and a similar effect of pactamycin, which inhibits initiation of translation, has been interpreted to indicate the coupling of translation and mRNA degradation (Linial et al., 1985). As *c-myc* mRNA contains a similar AU sequence (Figure 7), one might infer that there is a coupling between the degradation of mRNAs bearing the AU-rich sequences in their 3′ untranslated regions and the active translation of the messenger. Further experimentation is needed to address this issue.

Our experiments with T cells suggest that phorbol diester treatment may function to stabilize natural GM-CSF mRNAs that contain the AU sequence. The effect of phorbol diesters on cells is primarily the activation of protein kinase C (Arcoleo and Weinstein, 1985). Our observation would imply that protein phosphorylation can negatively modulate the pathway of mRNA degradation involving the AU sequence element. It will be interesting to examine the effect of phorbol diesters and other activators of protein kinase C on the stability of mRNAs containing the AU sequences. We note in this regard that Mitchell et al. (1985) have already demonstrated that TPA treatment of the monocytic cell lines U937 and HL-60 induces the stable expression of *c-fos* mRNA. However, interpretation of this result is complicated by the fact that the phorbol diester also caused the cells to differentiate into macrophages.

In conclusion, we believe the experiments we have

presented in this paper identify a new type of sequence element which we have strongly implicated as an mRNA destabilizer. The widespread occurrenc of similar sequence elements in the 3′ untranslated regions of transiently expressed mRNAs implies that the phenomenon we describe is of general significance, but this remains to be fully confirmed experimentally. We note in closing that the instability caused by the AU-rich element is a logical point for posttranscriptional regulation. Perhaps the transient increase in expression of certain proto-oncogene, lymphokine, and other immune mediator mRNAs, initiated by a signal received at the cell surface, results in part from a temporary block in specific mRNA degradation.

Experimental Procedures

Cell Culture and DNA Transfections

C10MJ-2, a human T lymphoblast cell line (Ayra et al., 1984; a gift from M. Chung) was maintained in RPMI medium, supplemented with 10% heat inactivated fetal calf serum. Cells were stimulated by addition of 0.3% phytohemagluttinin (PHA; Difco) or 5 ng/ml of the phorbol diester, 12-0-tetradecanoylphorbol-13-acetate (TPA). NIH3T3 and TSWOP cells (a derivative of NIH3T3, transformed by a temperature-sensitive mutant of polyoma virus DNA; a gift from C. Basilico, New York University) were propagated in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal calf serum (FCS). Approximately 10^6 cells were seeded per 90 mm culture dish, 24 hr prior to transfection. Five micrograms of plasmid DNA plus 15 μg of sonicated salmon sperm DNA were transfected by the calcium phosphate coprecipitation method (Graham and van der Eb, 1973; deVilliers and Schaffner, 1983). To obtain the pNEORβG^{AT}- and pNEORβG^{GC}-containing cell populations, TSWOP cells were transfected with linearized pNEORβG^{AT} or pNEORβG^{GC} DNA as described above. Colonies were selected in DMEM-10% FCS and 0.5 mg/ml G418 (Gibco) and pooled after 14 days.

Plasmid Construction

The plasmids pRβG^{AT} and pRβG^{GC} were made by insertion of synthetic DNA into the rabbit β-globin gene. Two pairs of complementary 58-mers were synthesized with an Applied Biosystem 380A DNA synthesizer. Upon annealing, these form the AT sequence of human GM-CSF (AT), and the control sequence with G's and C's interspersed (GC), flanked by 5′-GATC protruding termini. The synthetic oligomers were ligated to "pβGPy enhancer" DNA (de Villiers et al., 1982) partially cleaved with BglII. An oligonucleotide 20-mer spanning the junction was used as a probe in colony hybridization assays to identify plasmids with desired insertions and orientation. The regions containing the synthetic oligomer insertions were sequenced using the collapsed supercoil method of Chen and Seeberg (1985). The plasmid pNEORβG^{AT} was constructed using the BamHI–BstXI fragment of p4819 (a replication-defective polyoma virus DNA with an XhoI linker inserted in the origin of replication; a gift from S. Lupton), the PvuII–XhoI fragment of pRβG^{AT}, the EcoRI–BamHI fragment of pSV2NEO (Southern and Berg, 1981), and a synthetic XhoI–EcoRI adaptor. Plasmid pNEORβG^{GC} was then constructed by exchanging a 0.7 kb EcoRI–XhoI fragment of pNEORβG^{AT} with one of pRβG^{GC}.

DNA Probes

M13 clones were constructed to be used as templates for the generation of single-stranded DNA probes. β-globin: the PstI to BamHI fragment (453 bp) of a rabbit β-globin construct lacking the first intron (deVilliers et al., 1982; a gift from J. de Villiers) was inserted into M13mp8 and M13mp9. β_2 microglobulin: the 850 bp HindIII to EcoRI fragment of Ch4A. β_2-BALB (Parnes and Seidman, 1982) was subcloned into M13mp9. Neo: the 700 bp SphI to BglII fragment of SV2NEO (Southern and Berg, 1981) was inserted into M13mp9. GM-CSF: the 800 bp PstI to EcoRI fragment of the human GM-CSF gene (Wong et al., 1985) was subcloned into M13mp9.

RNA Preparation and Analysis

Total cytoplasmic RNA was prepared by the method of Favaloro et al., 1981. S1 nuclease mapping was done essentially as described previously (Berk and Sharp, 1977; Weaver and Weissmann, 1979). Single-stranded ^{32}P end-labeled DNA probes were made using M13mp9-based vectors containing the appropriate inserts as templates. ^{32}P end-labeled 20-mers (10^8 cpm per μg) complementary to the insert DNAs were annealed and elongated using the Klenow fragment (Ley et al., 1982). Elongated DNA was then cleaved at a distal restriction site, fractionated on a denaturing polyacrylamide gel (Maxam and Gilbert, 1980), and eluted from the gel. Northern blotting analysis was done as described (Seed, 1982; Thomas, 1980) using single-stranded uniformly labeled DNA probes with a specific activity of 10^9 cpm per μg prepared by primer extension of M13 clones in the presence of α-^{32}P-CTP. Nuclei were isolated according to Velcich and Ziff (1985) and used in nuclear run-on transcriptions assays as described by Linial et al. (1985).

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